

1 **Species indicators for naturally-regenerating and old calcareous grassland in southern**
2 **England**

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25

26 **Abstract**

27 Habitat restoration requires realistic goals. To naturally regenerate European lowland
28 calcareous grassland, whose extent has severely declined, over a century may be required for
29 vegetation to become indistinguishable from that of old calcareous grassland. Progress of
30 natural regeneration can be characterized using member species of the reference vegetation as
31 indicators of favourable site condition. Chronosequence studies have suggested that
32 calcareous-grassland species differ predictably in their ability to colonize ex-arable land, with
33 some usually colonizing early on, and others in later stages. If such patterns are affected by
34 gradually-attenuating establishment limitation, this would have important implications for
35 restoration practice and indication of progress. Particularly, late-colonizing species might be
36 better indicators of favourable site conditions than early colonizers.

37 To explore these aspects, we have reanalysed chronosequence data previously used to
38 investigate causal mechanisms affecting calcareous-grassland restoration progress. We
39 carried out an indicator species analysis to determine which species are indicative of
40 particular stages of natural regeneration. Using correlation analyses, we tested whether
41 species colonization patterns matched those found by previous chronosequence studies that
42 were geographically more limited or relied on more informal approaches to determine species
43 order of colonization. Correlation analyses were also used to test whether order of
44 colonization could be explained by establishment limitation or by dispersal limitation, or by
45 established plant strategies that underlie such limitations.

46 We identified 30 species as indicative of particular stages of natural regeneration, including
47 nine that specifically indicate old calcareous grassland. Correlation results confirmed high
48 congruence with species order of colonization in previous chronosequence studies, and
49 indicated that establishment limitation plays a role in shaping species order of colonization,
50 potentially mediated through differential stress tolerance. We failed to demonstrate a role of

dispersal limitation in shaping order of colonization. Based on our results, we derived three categories of indicator species for passively-restored calcareous grassland, mirroring the regeneration stage during which these species usually colonize. This includes a category labelled by us as ‘old-grassland indicators’ that achieve notable abundance only in old grassland. We conclude by discussing how such a categorization can benefit the measurement of restoration progress, the tentative identification of old grassland, and its conservation, e.g. through linking agri-environment payments to the occurrence of old-grassland indicators, positive change in farmer attitudes towards old grassland.

Keywords

calcareous grassland; dispersal limitation; establishment limitation; indicator species analysis; management continuity; natural regeneration; passive restoration

1. Introduction

Calcareous grassland is one of the most ancient (Bush and Flenley, 1987; Poschlod and WallisDeVries, 2002) and most species-rich (Wilson et al., 2012) semi-natural ecosystems in Europe. Exceptionally high botanical species richness in these grasslands has resulted from gradual species accumulation over long periods of continuous management (Aavik et al., 2008). However, during the last two centuries, various pressures, including afforestation, abandonment and agricultural intensification, have markedly reduced the overall extent of old calcareous grassland, both in continental Europe (van Dijk, 1991) and in the British Isles (Keymer and Leach, 1990). In Britain, reliable figures for the extent of this decline exist only for Dorset, where over 97% was lost between 1793 and 1983 (Keymer and Leach, 1990). Habitat loss still continues (Ridding et al., 2015), e.g. through land not under statutory protection being ploughed up once it comes out of agri-environment programmes (Pinches and Chaplin, 2014). In the UK, there are now only 40,000 ha of lowland calcareous grassland left (Bullock et al., 2011). Remaining sites can be small and isolated (Burnside et al., 2003; Hodgson et al., 2005; Polus et al., 2007). The effects of this fragmentation are exacerbated by widespread loss of traditional vectors of dispersal between sites, such as e.g. sowing of hayseed and movement of grazing animals between sites (Poschlod et al., 1998; Bruun and Fritzboøger, 2002; Auffret, 2011). As a result, colonization and extinction processes have been affected (Ozinga et al., 2009), and within-population genetic diversity reduced (Butaye et al., 2005).

Accordingly, lowland calcareous grassland is protected through the EC Habitats Directive (1992), listed in Annex I as habitat 6210 ('Seminatural dry grasslands and scrubland facies on calcareous substrates'). Moreover, efforts are underway to actively reverse some of the historic losses using habitat restoration and creation (Walker et al., 2004; Bullock et al., 2011), to reduce fragmentation and associated negative impacts. However, for

101 successful integration of ecological restoration into land management, restoration goals must
102 be realistic and attainable (Hobbs and Norton, 1996), taking into account the required
103 timescales. In calcareous grassland, these are long, with natural regeneration usually
104 requiring in excess of a century of continuous management (Gibson and Brown, 1991; Forey
105 and Dutoit, 2012), even in well-connected traditional landscapes with limited agricultural
106 improvement (Redhead et al., 2014).

107 Plant species composition at a restored site can be used in two different ways to assess
108 the degree to which restoration goals are met locally. First, at the level of the plant
109 community, floristic similarity with the reference community provides a direct measure of the
110 degree to which habitat creation has progressed (Ruprecht, 2006; Fagan et al., 2008). Second,
111 the presence and abundance of individual species also provides information on restoration
112 progress, e.g. serving as positive indicator species for environmental and management
113 conditions favourable for restoring the wider target community (for a definition of the
114 concept of 'positive indicator species' see Robertson and Jefferson, 2000).

115 For a species to be a suitable indicator for favourable habitat conditions, it must meet
116 several criteria. First, its occurrence should be reliably associated with such conditions, i.e. it
117 should only be present at sites where they are met (Öster et al., 2008). Thus, positive
118 indicator species for a target plant community usually are character species of that
119 community, with only limited occurrence in other communities (Bakker et al., 2000). Such an
120 approach is also used in the UK for evaluating the quality of existing semi-natural grassland
121 (Robertson and Jefferson, 2000; Joint Nature Conservation Committee, 2004). Furthermore, a
122 good indicator species should be reasonably common in the desired reference community,
123 present throughout the geographic range of interest, and easy to identify and obvious in the
124 sward for a reasonable time during the growing season (Robertson and Jefferson, 2000;
125 Wittig et al., 2006; Öster et al., 2008).

When restoring semi-natural calcareous grassland through natural regeneration, target species might differ with respect to the timeframe required for colonization. Some species usually colonize within a few years, and others only after decades (Gibson and Brown, 1991, 1992). Such patterns may not simply be a result of differential dispersal limitation, but also of differential establishment limitation, i.e. species may have differing capacities to establish successfully under the conditions prevalent on ex-arable land at a given time. One key factor may be the raised soil fertility on such land (Walker et al., 2004), resulting in amplified local-scale competitive interactions and limited opportunities for seedling establishment (Foster, 2001; Öster et al., 2009a). Such legacy effects are particularly pronounced at young sites (Hutchings and Booth, 1996a; Öster et al., 2009b; Horrocks et al., 2016), but can persist for decades (Öster et al., 2009b), potentially affecting order of target species colonization. Accordingly, even when target species are actively introduced, i.e. dispersal limitation is overturned, there are large and consistent species-level differences in establishment success (Pywell et al., 2003).

Hence, it might be argued that different target species may not be equal in their value as indicators of restoration progress. Those that tend to colonize later, at least if they do so due to establishment limitation, may be better indicators for favourable habitat condition than early-colonizing species. Thus, when defining indicator species for naturally regenerating grassland, the degree to which their colonization might initially be delayed due to establishment limitation should potentially be considered. On the other hand, if order of species colonization is primarily affected by dispersal limitation any recurring pattern in order of species colonization, rather than being a function of habitat quality, would instead reflect differences in species' dispersal capacity, with species better adapted to long-range dispersal typically colonizing earlier (Wagner, 2004).

150 All this has important implications for the practice of calcareous-grassland restoration
151 as well as for its assessment. Insights into the relative importance of establishment limitation
152 vs dispersal limitation during natural regeneration can inform suitable strategies for targeted
153 species introduction during active restoration. If establishment limitation is important, then a
154 ‘phased approach’ to restoration (Smith et al., 2008), with delayed sowing of those species
155 typically colonizing during later stages of natural regeneration, once a site has become
156 suitable for their establishment, may be more successful for establishing such species.
157 Furthermore, differential establishment limitation among colonizing target species would
158 have implications also with respect to evaluating progress of habitat condition towards that of
159 the target habitat, e.g. helping to inform payment levels in results-oriented agri-environment
160 schemes built around the occurrence of indicator species (Kaiser et al., 2010). Finally, due to
161 the scarcity of old reference grassland, and the fact that such old grassland can be destroyed if
162 it does not receive statutory protection, any species characteristic specifically of old
163 calcareous grassland would have particular relevance, as such species might help identify old
164 sites characterized by long management continuity, and linking AES remuneration with
165 occurrence of old-grassland indicators could boost farmers’ appreciation of their old
166 grassland.

167 Other recent chronosequence investigations of patterns of species colonization in
168 naturally regenerating calcareous grassland did not address such issues, and have either been
169 limited to a single area (Redhead et al., 2014), or were lacking proper statistical validation of
170 underlying colonization patterns (Gibson and Brown, 1991). Thus, to address these issues
171 based on a more generally applicable analysis of patterns of species colonization in naturally-
172 regenerating calcareous grassland, covering a representative range of sites across the whole
173 of lowland England, and of site ages ranging from four years of natural regeneration to old
174 calcareous grassland serving as reference, we re-analyzed a chronosequence dataset compiled

175 by Fagan et al. (2008). Our goals were to i) determine which plant species occurring during
176 the natural regeneration of lowland calcareous grassland in southern England are indicative of
177 particular stages of regeneration, with a particular focus on species indicative of old
178 grassland; ii) investigate whether species colonization patterns in our chronosequence match
179 those found in previous studies; iii) determine the extent to which species order of
180 colonization is affected by establishment limitation or dispersal limitation, and conforming
181 with established plant strategies thought to underlie such limitations. The focus on these goals
182 was chosen with respect to practical implications for the use of indicator species as outlined
183 above.

184

185 **2. Methods**

186 *2.1. Chronosequence data*

187 This study uses vegetation chronosequence data originally collected to explore how the use of
188 seed mixtures vs natural regeneration interacts with various environmental factors to affect
189 rates of progress of calcareous-grassland restoration on ex-arable land managed by grazing
190 after arable abandonment (Fagan et al., 2008). Fagan et al. (2008) had collected data from a
191 large number of restored sites and paired local reference sites of high quality old calcareous
192 grassland at least 200 years old at time of sampling. Here, we use data from those 16 sites in
193 their dataset that had naturally regenerated, along with data from an equal number of paired
194 local reference sites, objectively selected on the basis of being the closest ancient calcareous
195 grassland to their respective paired natural regeneration site that was characterized by a
196 similar slope angle and aspect (Fagan et al., 2008). Distance of a natural regeneration site to
197 the closest ancient calcareous grassland typically ranged from 0 m to about 1700 m. As
198 indicated by vegetation analyses using Tablefit 2.0 (Hill, 2015; results not shown), reference
199 sites fitted NVC lowland calcareous grassland communities CG2, CG3, CG4, and CG6 that

all occur in a similar climate space (Rodwell, 1992), with the more thermophilic CG1 and more continental CG7 communities not being represented. This sample covered five main calcareous hill ranges in lowland southern England (North Downs, South Downs, South Wessex Downs, Chilterns, and the Cotswolds; see Supplementary Fig. 1). All 32 sites were managed by grazing, in some instances supplemented by mowing. At the time of the field survey in summer 2004, the naturally-regenerated sites were between four and 60 years old. At each site, the field survey involved a visual estimation of percentage cover of vascular plant species in ten quadrats of 50 cm × 50 cm at 10-m intervals along a single linear transect. For further details on site selection and data recording see Fagan et al. (2008).

2.2. Data analysis

To determine species occurrence along our natural-regeneration chronosequence, the species cover data was analyzed using indicator species analysis (Dufrêne and Legendre, 1997). This was done using the ‘indicspecies’ R-package (De Cáceres and Jansen, 2015), which uses the modified indicator value index suggested by De Cáceres et al. (2008), defined as the square root of Dufrêne and Legendre’s (1997) IndVal index. Both the original and the modified index have been designed as a means to quantify and test the association between individual species and groups of sites, with the latter in our case being defined by site age since the onset of natural regeneration.

One of the advantages of both indices is that their values can be broken down into two multiplicative components, capturing different aspects: (1) A_{IND} , reflecting the relative abundance of a species within a target group of sites, compared to its abundance in other site groups, and (2) B_{PA} , indicating the frequency of occurrence of the species within that target group of sites. A_{IND} is a measure for the specificity of a species with respect to group

224 affiliation, and B_{PA} is a measure of the probability of finding the species at a site which is a
225 member of to the target group (Dufrêne and Legendre, 1997).

226 Before analysis, we averaged the cover values for each species across the ten replicate
227 quadrats per site. For analysis, we grouped sites according to age. Age classes were chosen to
228 minimize age ranges represented within each group, while ensuring that each group was
229 sufficiently large to allow statistical validation. We thus split the dataset into four distinct
230 primary site-age classes (Fig. 1), namely (a) young naturally regenerated (henceforth referred
231 to as age class Y; 4-8 years old at time of vegetation recording), (b) early medium-aged
232 naturally regenerated (age class M₁; 16-22 years old), (c) late medium-aged naturally
233 regenerated (age class M₂; 33-60 years old), and (d) old reference grassland (age class O;
234 > 200 years old). However, any fixed classification might not necessarily fit with the actual
235 temporal occurrence patterns of a given species, e.g. in our case, if a species typically occurs
236 in sites between four years old and 20 years old. In spite of the species occurring only in one
237 particular phase of natural regeneration, this pattern would not be picked up by an analysis
238 only using primary site-age classes. Hence, we also included five additional secondary site-
239 age classes (De Cáceres et al., 2010) in our analyses, each spanning two or three adjacent
240 primary site-age classes (as illustrated by braces in Fig. 1). Statistical testing was carried out
241 using permutation tests ($N = 99999$). As the three primary site-age classes of naturally
242 regenerating sites each contained fewer than ten sites, and the site age class coding for old
243 grassland contained as many sites as these three groupings together, we used non-equalized
244 indicator species analysis, to avoid giving undue influence to sites within the smaller
245 groupings (De Cáceres and Legendre, 2009).

246 To explore the level of congruence between the order in which, during natural
247 regeneration along our chrono-sequence, different indicator species for English lowland
248 calcareous grassland became indicative of a given regeneration stage, and the order in which

249 this occurred in previous chronosequence studies (Gibson and Brown, 1991; Redhead et al.,
250 2014), we carried out Spearman rank correlation. This rank-based approach is particularly
251 suitable here as we are using non-equidistant ordinal site-age classes, and requires no
252 assumptions to be met regarding the exact shape of investigated relationships. These analyses
253 were based on species ranks according to the 'youngest' primary age class for which each
254 given species was indicative in each respective chronosequence, also in the case of species
255 found to be indicative of two to three 'adjacent' primary age classes. For example, in our
256 chronosequence, if a species was indicative of natural regeneration stages classes M_1 and M_2 ,
257 its ranking was based on M_1 as the younger of the two age classes. Age class delimitations in
258 the previous chronosequence studies differed somewhat, but are nonetheless broadly
259 comparable. Gibson & Brown (1991) delimited three site-age classes, including young
260 naturally-regenerated sites up to ten years old, medium-aged sites 11-100 years old, and old
261 grassland sites over 100 years old (Gibson and Brown, 1991). They reported findings from
262 their 1989 field survey of seven naturally regenerated sites paired with old reference
263 grassland, along with the results of six previous studies by other researchers, with their
264 chronosequence thus based on a somewhat heterogeneous dataset, which did however cover a
265 relatively wide range of sites across southern England. However, their synthesis of results
266 across sites/studies (Table 1 in Gibson and Brown, 1991) did not involve formal statistical
267 validation. In contrast, Redhead et al.'s (2014) study focused on a single landscape, Salisbury
268 Plain, the largest continuous area of calcareous grassland in north-west Europe (Walker and
269 Pywell, 2000). Redhead et al. (2014) also delimited three site-age classes, including young
270 naturally-regenerated sites up to 12 years old, medium-aged sites 29-67 years old, and old
271 sites 116-157 years old. Species were assigned to natural regeneration stages according to
272 Chi-squared tests (Table S2 in Redhead et al., 2014).

273 We also carried out Spearman rank correlation to explore the extent to which
274 observed species order conformed with plant strategy theory, correlating species rank order
275 of colonization with species C-, S-, and R-scores as defined by Hunt et al. (2004) and listed in
276 Grime et al. (2007). Based on Grime's (2001) CSR theory, species with a high R-score
277 ('ruderals') should colonize a newly created fertile site such as recently abandoned arable
278 land very quickly, whereas species with a high S-score ('stress-tolerators'), due to their slow
279 growth and dispersal ability, are should be disadvantaged under such conditions against
280 ruderal and/or competitive species.

281 We also carried out Spearman rank correlations for the more limited set of species
282 identified by our analyses as component species of old calcareous grassland, but not
283 necessarily confined to this end stage of natural regeneration. These were done to explore
284 potential effects of establishment limitation and of dispersal limitation on the sequence of
285 colonization of these potential indicators of restoration progress. To explore the role of
286 establishment limitation, we correlated order of colonization of those such species that are
287 commonly sown in grassland restoration experiments with their index of establishment in the
288 first year after sowing as determined by Pywell et al. (2003). This index measures the
289 proportion of quadrats recorded that contained the species after it was sown, i.e. its values
290 range from zero to one, with higher values for species that are easier to establish during
291 restoration. Furthermore, these index values have been numerically adjusted for the fact that
292 not all species were sown in each experiment included in Pywell et al.'s (2003) analyses. To
293 explore the role of dispersal limitation we correlated order of colonization of all species
294 identified by our analyses as component species of old calcareous grassland with maximum
295 dispersal distances estimated from species traits and taxonomy using Tamme et al.'s (2014)
296 DispeRsal prediction tool. As our set of species contained several animal-dispersed species,
297 but no species specifically adapted to wind-dispersal, we used a model based on dispersal

298 syndrome, growth form, seed mass, seed release height, and plant taxonomy at the order-
299 level, but not taking into account seed terminal velocity, a trait whose usefulness does not
300 extend to animal-dispersed species (Tamme et al., 2014), and that contributes little to
301 explaining dispersal ability in calcareous grassland species (Diacon-Bolli et al., 2013). We
302 based estimation of maximum dispersal distances on trait data from the Kew Seed
303 Information Database (Liu et al., 2008; <http://data.kew.org/sid/>; seed mass), from the LEDA
304 plant-trait database (Kleyer et al., 2008; [http://www.uni-](http://www.uni-oldenburg.de/en/landeco/research/projects/LEDA)
305 [oldenburg.de/en/landeco/research/projects/LEDA](http://www.uni-oldenburg.de/en/landeco/research/projects/LEDA); release height), and from Grime et al.
306 (2007; dispersal syndrome and growth form). All correlation analysis as outlined above were
307 carried out using the base package in R v. 2.15.1 (R Core Team, 2012).

308 Further to our statistical analyses, to facilitate application of indicator species for
309 assessing naturally regenerating calcareous grassland, and for identifying old grassland sites,
310 we compiled a list of indicator species for to be used in condition assessments for
311 conservation purposes. We categorized the species on this list into early-stage and late-stage
312 indicator species of natural regeneration, along with indicator species specifically of old
313 calcareous grassland. While based on on our analytical results, the list neither includes
314 species with only limited occurrence during early regeneration, nor, in line with the
315 requirement for good indicator species to have only limited occurrence in non-target
316 communities (Bakker et al., 2000), does it include generalist grassland species known to
317 occur across a wide range of mesotrophic grasslands. We did however additionally include
318 several species of calcareous grassland that were underrepresented in our chronosequence,
319 but that on the basis of previous analyses by Gibson & Brown (1991) and by Redhead et al.
320 (2014) could be tentatively incorporated into this list with a reasonable expectation that a
321 similar analysis to our own, but based on a larger chronosequence dataset, should confirm
322 their tentative categorization. Finally, to explore the utility of the subset of indicator species

323 categorized as specific for old grassland, we carried out a graphic exploration, plotting
324 summed species richness, summed quadrat occupancy, and summed percentage cover of this
325 group of species against site-age class, to evaluate the potential discriminatory power of each
326 criterion with respect to separating old grassland from up to 60-year old naturally
327 regenerating grassland.

328

329 **3. Results**

330 *3.1. Species occurrence along our chronosequence*

331 Of the 136 species in our chronosequence dataset, 30 were identified by indicator species
332 analysis as indicative of particular stages during the natural regeneration of calcareous
333 grassland (Table 1). In 28 of these 30 species, the value of the A_{IND} component of the
334 indicator value is higher, and in many of these much higher, than that of the B_{PA} component,
335 illustrating that species temporal patterns during natural regeneration are primarily driven by
336 differential abundances between age classes, and that occurrence of these species is not
337 strictly confined to the site age classes they have been identified as being indicative of. Five
338 species were indicative of young to medium-aged naturally regenerating sites, being absent
339 from old calcareous grassland. The other 25 were all constituents of old calcareous grassland,
340 including seven that were indicative of old grassland and of naturally-regenerating grassland
341 at least 16 years old (i.e. age classes M_1 and M_2 along with age class O), and nine that were
342 indicative of old grassland and of naturally-regenerating grassland at least 33 years old (i.e.
343 age classes M_2 and O). The remaining nine indicator species were indicative specifically of
344 old calcareous grassland (Table 1).

345

346 *3.2. Comparison with other chronosequences*

Two-sided Spearman correlation showed that the lower limit of the site-age range for which species were indicative in our chronosequence was highly significantly correlated with the earliest site-age class in which the same species achieved notable abundance in Gibson & Brown's (1991) chronosequence ($r_s = 0.74$; $n = 25$; $P < 0.001$). Furthermore, the correlation between the lower limit of the site age-range for which species were indicative in our chronosequence and the lower limit of the site-age range in which species were typically well-represented in Redhead et al.'s (2014) chronosequence was also highly significant ($r_s = 0.65$; $n = 21$; $P = 0.001$).

355

356 3.3. Establishment limitation, dispersal limitation and CSR strategies

Spearman correlation indicated a strong negative relationship between the lower limit of the indicated site-age range of species for which this range included old grassland and Pywell et al.'s (2003) index of species performance in the first year after restoration sowing ($r_s = -0.65$; $n = 17$; $P = 0.005$). A similar correlation of the former parameter with estimated maximum dispersal distances of species based on their plant traits was not significant ($r_s = -0.23$; $n = 25$; $P = 0.271$).

For the complete set of 30 age-specific indicator species, also including early-successional species, we found a very strong positive correlation ($r_s = 0.82$; $n = 30$; $P < 0.001$) between lower limit of the site-age range for which species were indicative and their S-score *sensu* Grime (2001). We also found a strong negative correlation of species occurrence along the chronosequence with the R-score ($r_s = -0.70$; $n = 30$; $P < 0.001$), and a negative correlation with the C-score ($r_s = -0.45$; $n = 30$; $P = 0.014$), indicating that the more ruderal and/or competitive a species is, the more likely it achieves maximum representation during early natural regeneration.

371

3.4. Indicator species for condition assessment and for identification of old grassland

Excluding five species that are indicative specifically of early natural regeneration, along with two generalist species widely occurring also in mesotrophic grassland, 23 of the 30 'statistical' indicator species listed in Table 1 are potentially useful as positive indicators *sensu* Robertson and Jefferson (2000). A further nine species were identified as potentially useful from the two previous chronosequence studies (Gibson and Brown, 1991; Redhead et al., 2014), resulting in 32 positive indicator species that were assigned to three groups (Table 2), depending on being high abundance i) already in the early intermediate stages of natural regeneration (= 'early-stage indicators'), ii) from the late intermediate stages of natural regeneration (= 'late-stage indicators') onwards, or iii) only in fully-formed 'old' calcareous grassland (= 'old-grassland indicators').

As shown in Fig. 2a, which graphically explores the discriminatory power of summed species richness, summed quadrat occupancy, and summed percentage cover of positive indicator species of old grassland as listed in Table 2, these criteria differ in the degree of overlap among value ranges for different site-age classes in general, and between late-stage natural regeneration and old grassland in particular. The overlap was strongest for summed species richness, but less pronounced for summed quadrat occupancy (Fig. 2b), and in particular for summed percentage cover (Fig. 2c).

4. Discussion

4.1 Species indicators for naturally-regenerating sites

Using chronosequence data collected by Fagan et al. (2008) across southern England, we statistically validated 30 plant species as indicative of particular stages of natural regeneration towards an endpoint of high-quality lowland calcareous grassland. Twenty-five of these species are characteristic of high-quality calcareous grassland, with 16 typically achieving

397 their characteristic abundance already in the mid-stages of natural regeneration. For 17 of
398 these species, Pywell et al. (2003) had calculated indices characterizing establishment success
399 when sown during ecological restoration. The highly significantly negative correlation
400 between indicated order of colonization for these species and Pywell et al.'s (2003) index of
401 first-year performance after sowing strongly suggests that species that are slower at achieving
402 appreciable representation during natural regeneration are more difficult-to-establish even
403 when not dispersal-limited. In other words, those restoration target species whose occurrence
404 in our chronosequence tends to be most strictly limited to ancient grassland appear to be
405 characterized by stricter requirements with respect to their realized niche, and stronger
406 establishment limitation, compared to earlier-colonizing species. This interpretation is further
407 supported by the strong positive correlation between species order of colonization and their
408 stress tolerance score *sensu* Grime (2001), suggesting that slow-growing specialist species of
409 calcareous grassland are not well adapted to immediately colonize young restoration sites
410 characterized by high residual fertility.

411 Although it has been shown previously that characteristic plant species can be absent
412 from old grassland simply because of their poor dispersal (Riibak et al., 2015), we found no
413 correlation between order of colonization of species characteristic for calcareous grassland
414 and their estimated maximum dispersal distance. However, while our results clearly indicate
415 an important role of gradually-attenuating establishment limitation in shaping the order of
416 colonization even in the mid to late stages of natural regeneration, they cannot be taken as
417 proof that the dispersal characteristics of species are irrelevant. Our simple correlation of
418 species colonization order with estimated maximum dispersal distance did not take into
419 account actual distances that colonizing species had to cover to reach each respective
420 naturally regenerating site, as this information was not available. Nonetheless, our results
421 appear to suggest that, at least for the group of late-successional characteristic species of

422 calcareous grassland, dispersal ability may not be an overriding factor in determining their
423 order of colonization. This is also underlined by the fact that unassisted dispersal of the vast
424 majority of specialist species of calcareous grassland, and among these particularly forbs,
425 tends to be poor (Hutchings and Booth, 1996b; Diacon-Bolli et al., 2013).

426 The fact that order of target species colonization appears to be at least partly determined by
427 differential establishment limitation has important implications for the issue of species'
428 equivalence as indicators of favourable site conditions for restoration of the target vegetation.
429 It suggests that occurrence at a given restoration site of a calcareous-grassland specialist
430 species known to reach relatively high abundance mainly in older grassland might be a better
431 indication of highly favourable site conditions than the occurrence of a different species of
432 calcareous grassland that tends to achieve characteristic levels of abundance relatively earlier
433 on during natural regeneration. Thus, the former should be given more weight when assessing
434 favourability of site conditions.

435 The classification of 30 indicator species of regenerating and old calcareous grassland
436 derived here is confirmatory of the findings of Gibson and Brown (1991) who classified
437 species more informally into site-age groups, and of Redhead et al. (2014) whose
438 investigation was limited to a single landscape. However, our classification is improved, as it
439 is built on rigorous statistical analyses of a dataset with wide geographic coverage.

440 As already stated in the Introduction, for a species to be a good indicator for a given
441 type of vegetation, it should be characterized by only limited occurrence in other types of
442 vegetation (Bakker et al., 2000). However, two of the species recorded in our chronosequence
443 that were validated as indicative also of old calcareous grassland, *Dactylis glomerata* and
444 *Holcus lanatus*, are generalist grasses characterized by a particularly wide ecological
445 amplitude across a wider range of grassland types not covered by our study. These species are
446 thus not suitable as indicators for characterizing progress of natural regeneration towards

447 high-quality calcareous grassland. On the other hand, other species that likely are suitable
448 indicators for such conditions have been underrepresented in our dataset. Thus, in addition to
449 the 23 species remaining after exclusion of *Dactylis* and *Holcus*, based on the findings of the
450 two previous chronosequence studies (Gibson and Brown, 1991; Redhead et al., 2014), a
451 further nine have been assigned tentatively to one of the three groups of positive indicators
452 (Table 2). One of these additionally included species is *Festuca ovina* agg. which in Fagan et
453 al.'s (2008) study was recorded as a species aggregate together with *Festuca rubra*, and
454 hence could not be separately analysed for its indicator value here. In total, we were able to
455 classify 32 specialist species of calcareous grassland as positive indicators for naturally
456 regenerating grassland, all of which are obvious in the vegetation for a reasonable time
457 during the growing season, and widespread in lowland calcareous grassland across southern
458 England (Preston et al., 2002). Table 2 gives an indication of which of these species are used
459 in England's current Countryside Stewardship scheme as positive indicators for extant semi-
460 natural lowland calcareous grassland habitat of principal importance (Natural England, 2016).
461 As can be seen from this information, for reasons of botanical identifiability, species typically
462 used for condition assessments in such schemes are mostly broadleaf species, excluding
463 Poaceae, but including *Carex caryophyllea* and *C. flacca* within a group of 'small blue-green
464 sedges' (Natural England, 2016). Accordingly, inclusion of the grasses listed in Table 2 here
465 in any condition assessments would be contingent on the use of botanically-experienced
466 surveyors.

467 When using indicator species for evaluating progress of natural regeneration towards
468 mature lowland calcareous grassland, one should consider differences among species in the
469 ability to colonize sites early on. As underlined by our analyses, this ability depends at least
470 partially on habitat specialization. This means that the presence of a late-colonizing species
471 requiring habitat conditions more similar to those of mature calcareous grassland should be

472 given more weight than that of an early-colonizing species whose realized niche requirements
473 are less strict. Based on the classification in Table 2, this could be done by giving most
474 weight to the presence of species categorized as ‘old-grassland indicators’, and least weight
475 to that of ‘early-stage indicators’. Another reason for doing so is that, of the nine species that
476 were classified as early-stage indicators, six – *Leucanthemum vulgare*, *Lotus corniculatus*,
477 *Plantago lanceolata*, *Prunella vulgaris*, *Ranunculus bulbosus* and *Trisetum flavescens* – are
478 rather widespread (Preston et al., 2002), and also occur in neutral mesotrophic grassland
479 (Rodwell, 1992; Hill et al., 2004). Most of the species in the other two categories are more
480 exclusive to calcareous grassland. However, it should be noted that *Brachypodium pinnatum*
481 and *Bromopsis erecta*, are considered indicators for deficient grazing in calcareous grassland
482 when occurring at very high abundance (Hall, 1971; Harper, 1971; Wells and Wells, 1974).

483

484 4.2 Species indicators of old calcareous grassland

485 Our findings do also have implications for the conservation of well-established calcareous
486 grassland, e.g. in terms of site prioritization. Even in well-connected landscapes in which
487 such grasslands are traditionally grazed, more than a century is required for calcareous
488 grassland to regenerate after arable land-use (Redhead et al., 2014). During long continuous
489 management, specialist plant species gradually accumulate (Röder et al., 2006; Aavik et al.,
490 2008), resulting in the characteristically high small-scale richness of old calcareous grassland
491 (Pärtel and Zobel, 1999). However, old calcareous grassland also represents an invaluable
492 habitat for other biota including grassland butterflies (van Swaay, 2002), other arthropods
493 (McLean, 1990), and fungi (McHugh et al., 2001; Griffith et al., 2002), and its conservation
494 benefits the preservation of other characteristics of traditionally-managed ecosystems such as
495 e.g. undisturbed soil profiles.

496 If site age remains unaccounted for in conservation inventories of the grassland
497 resources, the loss of old grassland of outstanding botanical quality could go unnoticed. For
498 example, while an assessment of semi-natural montane grassland in a German region
499 indicated increased availability of good-quality montane grassland, additional assessments
500 based on ancient maps indicated a marked loss of species-rich old sites (Waesch and Becker,
501 2009). To prevent the loss of valuable old grassland, it must be considered separately.
502 However, while management continuity has already been included in monitoring efforts, e.g.
503 in Sweden (Ihse and Lindahl, 2000; Johansson et al., 2008), there will not always be a
504 continuity of maps or other records to prove site age. The species that we have identified as
505 old-grassland indicators, at least in the absence of active species introduction, are
506 characteristic of such old sites. Similar to poor-colonizing species of the woodland ground
507 flora that are indicators of forest continuity (Peterken, 1974; Hermy et al., 1999), these late
508 colonizers could be used as indicators of old calcareous grassland, to help identify non-
509 statutory sites with long-term management continuity, and deserving of conservation priority.
510 However, as shown by the considerable vertical overlap of the data scatter between age
511 classes O and M₂ in Fig. 2a, a simple count of old-grassland indicators at a site is not
512 sufficient for distinguishing ancient grassland from naturally-regenerating grassland only a
513 few decades old. Summed total cover of such species is much more suitable for
514 distinguishing these two types of grassland (Fig. 2c), with the implication that the high
515 indicator value of these species for old grassland is mainly a function of their local
516 abundance. In our chronosequence, up to six old-grassland indicator species were found in
517 33- to 60-year old naturally-regenerating grassland (= age class M₂), whereas old grassland
518 sometimes had only two such species recorded (Fig. 2a). Summed cover of old-grassland
519 indicators, and to a lesser extent summed frequency of quadrat occurrences, appear to be
520 more suitable criteria for identifying old grassland (Fig. 2b and 2c). In the case of summed

cover, all but two sites of old grassland were characterized by a total cover of old-grassland indicators greater than 10%, whereas none of the sites of age class M₂ reached this threshold. Thus, a practical application of indicator species for old calcareous grassland with long management continuity should preferably use cover- or frequency-based criteria. In addition to site evaluation, this could also e.g. include linking payments for agri-environment grassland conservation options with the prevalence of indicator species of old grassland, i.e. by paying higher rates for grassland assessed to be potentially older. Current schemes such as England's Countryside Stewardship scheme do not make any distinction with respect to typical time to colonization for different indicator species, which means early colonizers such as e.g. *Lotus corniculatus* and late-colonizing indicators of old calcareous grassland such as e.g. *Carex caryophylla* and *Helianthemum nummularium* are treated similarly (Table 2). However, a high frequency and cover of old-grassland indicators does not conclusively prove a site is centuries old without any history of disturbance. Similar botanical conditions are also found in calcareous grassland temporarily ploughed up during World War II (e.g. Cornish, 1954), where land-use had been altered for only a short period, thus allowing for quick recolonization by calcareous-grassland species from the surroundings and from the soil seed bank. In such instances, disturbance impacts on the biotic integrity of a site would usually have been quite limited. In such situations, to further ascertain biotic integrity and land use history, it may also be possible to take into account other indicators, e.g. presence of certain grassland fungi (Rotheroe et al., 1996), or density and size of *Lasius flavus* ant-hills (King, 1981).

Regardless of the challenges associated with identifying old grassland sites, an increased awareness of the particularly high conservation value of such sites and of their associated biodiversity, as e.g. conveyed through a linking of AES payments with the occurrence of suitable indicator species, might help induce a positive change in the attitude of

546 farmers and the wider public towards such sites (Ihse and Lindahl, 2000), and enhance their
547 perceived heritage value. Indicator species of grassland management continuity, as identified
548 by our study for lowland calcareous grassland, could be useful in this context.

549

550 **Acknowledgements**

551 This study was funded by Defra (project BD5101) and builds on a NERC-funded PhD project
552 carried by Kate Fagan. We thank Christina Lindahl and Per Milberg for stimulating
553 discussions on ancient grasslands, and Riin Tamme for advice on how to use the DispeRsal
554 tool. We also thank the referees for constructive comments.

555

556 **Appendix A. Supplementary data**

557 Supplementary data to this article can be found online.

558

559

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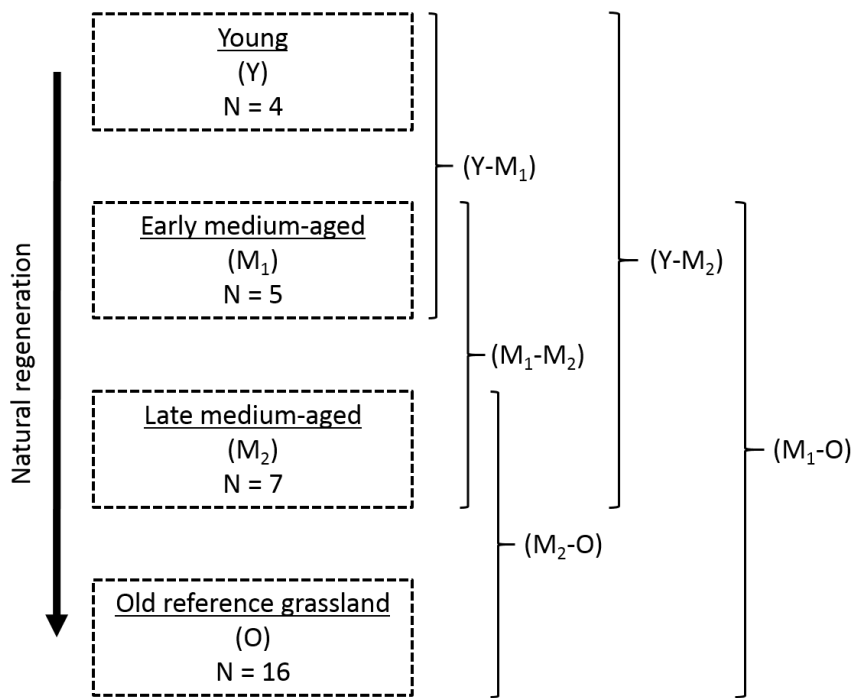
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791 Figure 1. Site classification of field sites as used in indicator species analysis. Primary site-
792 age classes are young naturally regenerated (Y; 4-8 years old with individual site ages of
793 4,5,7 and 8 years old), early medium-aged naturally-regenerated (M₁; 16-22 years old with
794 individual site ages of 16, 17, 18, 20 and 22 years old), late medium-aged naturally-
795 regenerated (M₂; 33-60 years old with individual site ages of 33, 40, 46, 47, 55, 58 and 60
796 years old) and old reference grassland (O; all site ages > 200 years old). Additional secondary
797 site-age classes also included in the analysis, spanning two or three adjacent primary age
798 classes, are indicated by braces.

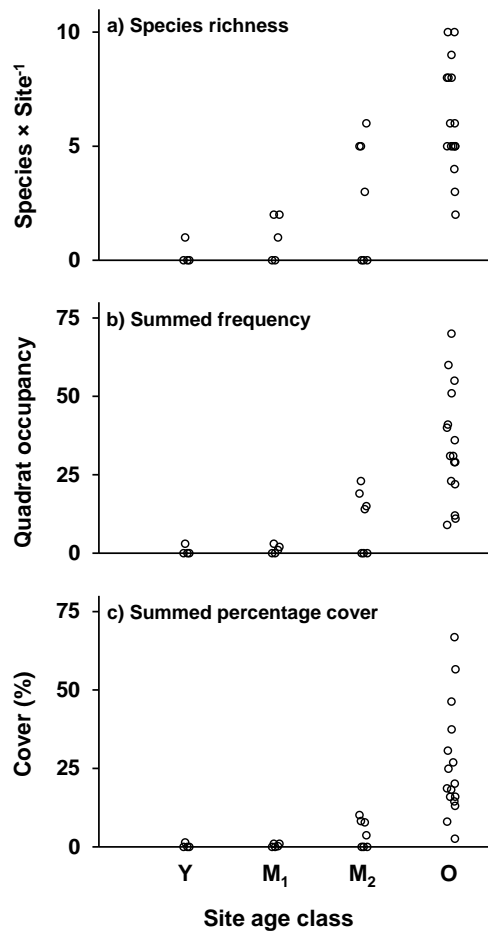


Figure 2. Scatter plots of a) old-grassland indicator site-level species richness, b) summed quadrat frequency, and c) summed percentage cover in 32 naturally regenerating and old grassland sites included in our study. Values are based on the occurrence of old-grassland indicator species identified by our study, not including *Festuca ovina*, which was recorded as part of a species aggregate with *F. rubra* by Fagan et al. (2008). Summed frequency values represent the total occurrence across species in the ten small quadrats recorded per site. Because of multiple overlaying data points, a small amount of horizontal jitter was applied.

825 Table 1. Results of indicator species analyses based on species mean cover at 32 grassland sites, including 16 naturally regenerating sites and 16
826 reference sites of old grassland. Along with the indicator age range, indicator values (IV), P values based on permutation tests ($N = 99999$), and
827 the two multiplicative components of the IV, A_{IND} and B_{PA} are listed, with A_{IND} reflecting the relative abundance in terms of cover of the
828 respective species within the group of sites of the indicated age range, and B_{PA} indicating the frequency of occurrence within this group of sites.
829 For comparison, the indicated site-age range is also provided according to two other studies (Gibson and Brown, 1991; Redhead et al., 2014).
830 Site-age categories used in our indicator species analysis were defined slightly differently from those used in the other two studies, but are
831 broadly comparable. In our study, Y: 4-8 years old; M1: 16-22 years old; M2: 33-60 years old; O: ancient grassland >200 years old; in Gibson &
832 Brown (1991), Y: up to 10 years old; M: 11-100 years old; O: ancient grassland >100 years old; in Redhead et al. (2014), Y: 0-12 years old; M:
833 29-67 years old; O: ancient grassland 116-157 years old. Habitat preference for calcareous grassland (Hill et al., 2004), CSR strategies (Grime et
834 al., 2007), and a performance index of species establishment in the first year after sowing at multiple UK restoration sites (Pywell et al., 2003)
835 are provided. The values of this performance index range from 0 to 1, with low values indicating typically poor establishment, and high values
836 indicating typically reliable establishment. For habitat preference, 'yes' indicates that a species has a preference exclusively for calcareous
837 grassland.

	Indicator species analysis based on Fagan et al. (2008)					Site age range in other studies				
Species	IV	P	A _{IND}	B _{PA}	Indicator age range	Gibson and Brown (1991)	Redhead et al. (2014)	Calcareous grassland habitat preference (Hill et al. 2004)	CSR type (Grime et al. 2007)	Index of first-year performance after sowing (Pywell et al. 2003)
<i>Geranium columbinum</i>	0.59	0.038	0.70	0.50	Y	-	-	No	R/SR	-
<i>Taraxacum officinale</i> agg.	0.72	0.006	0.69	0.75	Y	Y-M	Y-M	No	R/CSR	-
<i>Tragopogon pratensis</i>	0.62	0.036	0.64	0.60	M ₁	-	M	No	CR/CSR	-
<i>Agrostis stolonifera</i>	0.83	0.006	0.92	0.75	Y-M ₂	Y-M	Y	No	CR	-
<i>Potentilla reptans</i>	0.69	0.040	0.95	0.50	M ₁ -M ₂	Y-M	M	No	CR/CSR	-
<i>Avenula pratensis</i>	0.92	0.008	1.00	0.86	M ₁ -O	M-O	O	Yes	SC/CSR	-
<i>Dactylis glomerata</i>	0.95	0.013	0.98	0.93	M ₁ -O	M	Y	No	C/CSR	-
<i>Holcus lanatus</i>	0.97	0.031	0.98	0.96	M ₁ -O	Y-M	-	No	CSR	-
<i>Lotus corniculatus</i>	0.96	0.003	0.99	0.93	M ₁ -O	M-O	-	No	S/CSR	0.21
<i>Prunella vulgaris</i>	0.90	0.022	0.95	0.86	M ₁ -O	Y-M	-	Np	CSR	0.19
<i>Ranunculus bulbosus</i>	0.80	0.034	1.00	0.64	M ₁ -O	M-O	M	No	SR	0.11
<i>Trisetum flavescens</i>	0.92	0.014	0.98	0.86	M ₁ -O	Y-M	-	No	CSR	0.65
<i>Briza media</i>	0.86	< 0.001	1.00	0.74	M ₂ -O	M-O	M-O	Yes	S/CSR	< 0.01
<i>Bromopsis erecta</i>	0.74	0.044	0.97	0.57	M ₂ -O	O	-	Yes	SC/CSR	0.12
<i>Euphrasia officinalis</i> agg.	0.75	0.018	0.99	0.57	M ₂ -O	Y-M	M	No	SR	-
<i>Galium verum</i>	0.90	< 0.001	0.97	0.83	M ₂ -O	O	-	Yes	SC/CSR	0.05
<i>Linum catharticum</i>	0.86	0.001	0.95	0.78	M ₂ -O	M-O	M-O	Yes	SR	< 0.01
<i>Pimpinella saxifraga</i>	0.75	0.034	0.98	0.57	M ₂ -O	M-O	-	Yes	SR/CSR	0.01
<i>Thymus polytrichus</i>	0.78	0.005	1.00	0.61	M ₂ -O	M-O	O	No	S	0.02
<i>Viola hirta</i>	0.78	0.005	1.00	0.61	M ₂ -O	M-O	M-O	Yes	S/CSR	0.01
<i>Scabiosa columbaria</i>	0.75	0.009	1.00	0.57	M ₂ -O	-	O	Yes	S/SR	0.01
<i>Asperula cynanchica</i>	0.65	0.041	0.98	0.44	O	O	M-O	Yes	-	-
<i>Avenula pubescens</i>	0.79	0.008	0.83	0.75	O	-	-	No	S/CSR	-
<i>Campanula rotundifolia</i>	0.70	0.022	0.88	0.56	O	-	O	Yes	S/CSR	< 0.01
<i>Carex caryophylla</i>	0.61	0.041	1.00	0.38	O	O	O	Yes	S	-
<i>Carex flacca</i>	0.93	< 0.001	0.86	1.00	O	M-O	O	No	S	-
<i>Filipendula vulgaris</i>	0.66	0.028	0.99	0.44	O	O	O	Yes	S/CSR	< 0.01
<i>Helianthemum nummularium</i>	0.66	0.029	1.00	0.44	O	O	O	Yes	S	< 0.01
<i>Koeleria macrantha</i>	0.75	0.005	0.91	0.62	O	O	M-O	Yes	S	0.08
<i>Poterium sanguisorba</i>	0.93	< 0.001	0.92	0.94	O	M-O	O	Yes	S/CSR	0.04

847

848

849 Table 2. Suggested list of indicator species of calcareous grassland, subdivided into early-stage indicators and late-stage indicators for natural
850 regeneration, and old-grassland indicators. This list includes 23 of the 25 species shown in Table 1 to be statistically indicative of a site-age
851 range that includes old grassland and a further nine species marked with ‘(t)’ that have been included tentatively on the basis of having been
852 underrepresented in our chronosequence while having shown show clear site-age preferences in Gibson and Brown (1991) and Redhead et al.
853 (2014). Early-stage indicators achieve notable abundance after 10-30 years of natural regeneration, late-stage indicators after 30-60 years, and
854 old-grassland indicators often only after 100 years or more. Species listed in Natural England (2016) as positive indicators for the condition of
855 extant lowland calcareous grassland in England’s current Countryside Stewardship scheme are marked with a ‘+’.

856

Early-stage indicators	Late-stage indicator	Old-grassland indicators
<i>Anthyllis vulneraria</i> ⁺ (t)	<i>Briza media</i>	<i>Asperula cynanchica</i> ⁺
<i>Avenula pratensis</i>	<i>Bromopsis erecta</i>	<i>Avenula pubescens</i>
<i>Leucanthemum vulgare</i> ⁺ (t)	<i>Cirsium acaule</i> ⁺ (t)	<i>Brachypodium pinnatum</i> (t)
<i>Lotus corniculatus</i> ⁺	<i>Clinopodium vulgare</i> ⁺ (t)	<i>Campanula rotundifolia</i> ⁺
<i>Ononis repens</i> (t)	<i>Euphrasia officinalis</i> agg. ⁺	<i>Carex caryophyllea</i> ⁺
<i>Plantago lanceolata</i> (t)	<i>Galium verum</i> ⁺	<i>Carex flacca</i> ⁺
<i>Prunella vulgaris</i>	<i>Linum catharticum</i> ⁺	<i>Festuca ovina</i> agg. (t)
<i>Ranunculus bulbosus</i>	<i>Pimpinella saxifraga</i> ⁺	<i>Filipendula vulgaris</i> ⁺
<i>Trisetum flavescens</i>	<i>Scabiosa columbaria</i> ⁺	<i>Helianthemum nummularium</i> ⁺
	<i>Thymus polytrichus</i> ⁺	<i>Hippocrepis comosa</i> ⁺ (t)
	<i>Viola hirta</i> ⁺	<i>Koeleria macrantha</i>
		<i>Poterium sanguisorba</i> ⁺

857

Species indicators for naturally-regenerating and old calcareous grassland in southern

England

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Supplementary Information:



Supplementary Figure 1. The locations of 16 natural regeneration sites, indicated by '•', and of their paired ancient grassland reference sites, indicated by '+', across five calcareous hill ranges in lowland southern England. The lighter grey colour indicates the occurrence of calcareous soils.